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Induction of Prophage Lambda by Chlorinated Pesticides (Journal Version)

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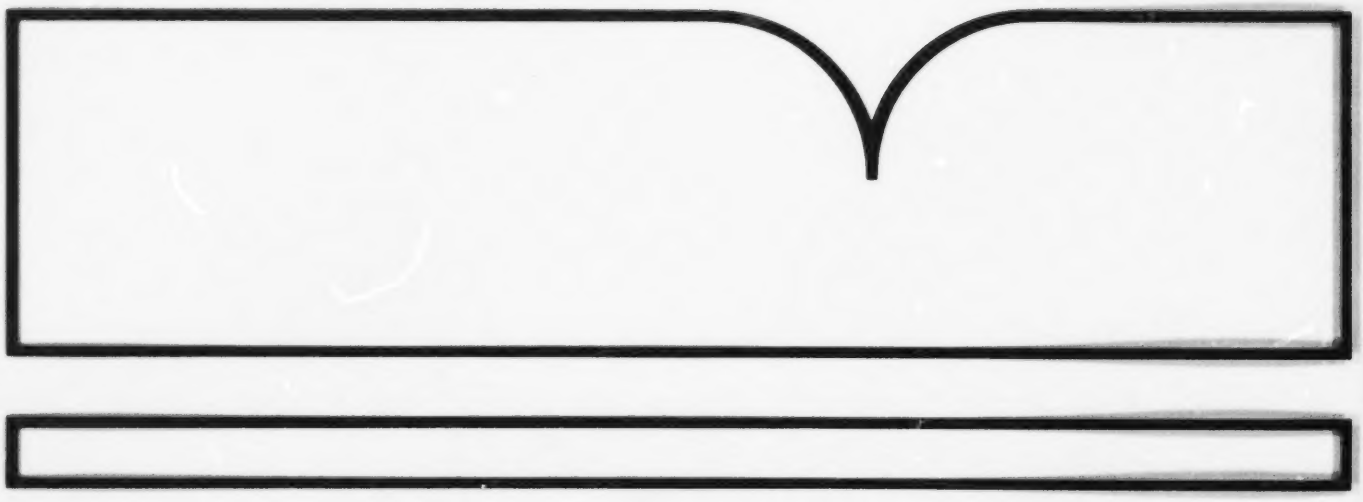

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16. ABSTRACT

Chlorinated organics represent an important class of environmental carcinogens. However, only a small percentage of the carcinogens of this chemical class are genotoxic in prokaryotic bioassays such as the Salmonella assay. In an effort to identify a short-term assay sensitive to chlorinated carcinogens, we have tested a group of chlorinated pesticides, most of which are carcinogenic in rodents, in a prophage-induction assay developed by Rossman et al. (1984). The Microscreen phage-induction assay is a rapid, inexpensive, miniaturized system that uses the induction of prophage λ in *Escherichia coli* as an indicator of genetic damage. It has been used successfully to screen complex environmental samples for genotoxicants and has detected carcinogenic metals that are refractory in the Salmonella assay. The pesticides tested were malathion, monuron, *p,p'*-DDT, mirex, lindane, nitrofen, chlordane, toxaphene, captan, and dichlorvos. All but the first 4 induced prophage. The remaining pesticides were ranked as follows according to induction potency in the presence of S9: captan > dichlorvos > toxaphene > lindane > nitrofen > chlordane. Rankings were similar in the absence of S9. Of these 6 pesticides, only nitrofen required S9 to induce prophage. Comparisons with mutagenesis data in Salmonella indicated that the Microscreen assay detected as genotoxic each of the pesticides that were mutagenic in Salmonella; moreover, it detected 2 additional carcinogens (chlordane and lindane) that were not mutagenic in the Salmonella assay. The possible use of the Microscreen phage-induction assay to detect chlorinated organics is discussed.

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Induction of prophage lambda by chlorinated pesticides

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Summary

Chlorinated organics represent an important class of environmental carcinogens. However, only a small percentage of the carcinogens of this chemical class are genotoxic in prokaryotic bioassays such as the Salmonella assay. In an effort to identify a short-term assay sensitive to chlorinated carcinogens, we have tested a group of chlorinated pesticides, most of which are carcinogenic in rodents, in a prophage-induction assay developed by Rossman et al. (1984). The Microscreen phage-induction assay is a rapid, inexpensive, miniaturized system that uses the induction of prophage λ in *Escherichia coli* as an indicator of genetic damage. It has been used successfully to screen complex environmental samples for genotoxicants and has detected carcinogenic metals that are refractory in the Salmonella assay. The pesticides tested were malathion, monuron, *p,p'*-DDT, mirex, lindane, nitrofen, chlordane, toxaphene, captan, and dichlorvos. All but the first 4 induced prophage. The remaining pesticides were ranked as follows according to induction potency in the presence of S9: captan > dichlorvos > toxaphene > lindane > nitrofen > chlordane. Rankings were similar in the absence of S9. Of these 6 pesticides, only nitrofen required S9 to induce prophage. Comparisons with mutagenesis data in Salmonella indicated that the Microscreen assay detected as genotoxic each of the pesticides that were mutagenic in Salmonella; moreover, it detected 2 additional carcinogens (chlordane and lindane) that were not mutagenic in the Salmonella assay. The possible use of the Microscreen phage-induction assay to detect chlorinated organics is discussed.

The Salmonella mutagenicity assay has been used more extensively than any other short-term bioassay for the purpose of screening environmental samples and complex mixtures for mutagenic activity (Waters et al., 1985). Although the assay is sensitive to most classes of genotoxic compounds, there are some chemical classes of environmental carcinogens such as chlorinated organics that are not detected well by this assay (McCann et al., 1975; Zeiger and Tennant, 1986). Yet chlorinated organics are an important component of many environmental pollutants, including industrial chemicals, hazardous wastes and effluents (Rich, 1986), and agricultural chemicals (Garrett et al., 1986). The prevalence and persistence of carcinogenic chlorinated organics in the environment un-

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S9 mix

Rat liver homogenate (S9) was prepared from male Sprague–Dawley rats injected with Aroclor 1254 as described by Maron and Ames (1983). The same batch of S9 (24.75 mg protein/ml) was used for all experiments reported here. The co-factor mix contained per 5 ml: 3.25 ml of sodium phosphate buffer (pH 7.4, 0.2 M); 0.125 ml of a solution containing 0.4 M $MgCl_2$ and 1.65 M KCl; 3.1 mg of NADP; 3.7 mg of G-6-P; 1.5 ml of dH_2O; and 0.125 ml of S9. This gave a final concentration of 2.5% S9 in the S9 mix as recommended by Rossman et al. (1985).

Preparation of microtiter plates

Plastic, sterile microtiter plates with 96 wells (Corning Laboratory Sciences Co.) were used for the microsuspension assay. The top row of 12 wells received 250 μl of supplemented minimal medium and 50 μl of the test compound dissolved in acetone or 50 μl of the control. The remaining wells received 150 μl of the medium. Two-fold serial dilutions of the agents (150 μl : 150 μl) were then made down the columns of the plate. Dilutions were carried routinely into a second plate to assure identification of the dose range. Each well was inoculated with 75 μl ($\sim 2 \times 10^6$ cells) of the resuspended log-phase culture of $WP2_s(\lambda)$ and 25 μl of either S9 mix or supplemented minimal medium. For tests with S9, the positive control was 2-AA at a starting concentration of 1.25 μg/well; for tests without S9, the positive control was 2-NF at a starting concentration of 75 μg/well. Acetone was the negative control with and without S9.

After the contents of the microtiter plates were mixed (Minimixer from Fisher Scientific), the plates were enclosed in individual plastic zip-lock bags to reduce evaporation and incubated overnight (20 h) at 37°C. The relatively lengthy incubation period allowed exposure to occur over many cell generations. After incubation, wells were scored for turbidity, with turbid wells indicating cell growth and clear wells indicating cytotoxicity and/or inhibition of cell growth. Agents were tested at increasing stock concentrations until the limits of solubility were reached or until cytotoxicity was observed.

Assay for plaque formation

An adaptation of the method of Rossman et al. (1984, 1985) was used to assay for plaque formation. The substitution of NaCl for $MgSO_4$ in the top agar simplified preparation in our laboratory and had no effect on the observed frequency of induction. To enhance plaque morphology, half-strength tryptone medium (5 g of Bacto tryptone, 2.5 g of NaCl, 15 g of Bacto agar, and 1 l of dH_2O) (Arber et al., 1983) was substituted for half-strength Luria broth for the bottom agar of the plaque assay plates.

An aliquot of 100 μl from selected wells of the microtiter plate was diluted 100-fold in supplemented minimal medium, and chloroform was added to the dilution tubes to kill viable lysogens. To ~ 2.5 ml of molten top agar (0.65% Bacto agar and 10 mM NaCl) were added 200 μl of an overnight culture of SR714 and either 50 μl (+S9) or 500 μl (−S9) of the diluted prophage. The two dilution schemes described here were used to obtain similar numbers of background plaque-forming units (PFU) per plate in the presence and absence of S9. We have observed that some batches of S9 increase the background PFU/plate compared to the background obtained in the absence of S9 when a constant concentration of 2.5% S9 is used.

The contents of the tubes were vortexed and poured onto plates containing half-strength tryptone medium. Plates were incubated overnight at 35°C. At this temperature, large, clear plaques with well-defined edges were produced; at 37°C, the background lawn overgrew the plaques, resulting in small, turbid plaques that were difficult to quantitate.

Plaques were counted by hand. Currently, however, we are counting plaques by means of a modified BioTran III automatic colony counter from New Brunswick Scientific Co., Edison, NJ. We felt that this recent improvement was worth mentioning due to the relative speed with which plaques can now be counted. The dilution tubes were sampled in duplicate, and all experiments were repeated at least twice.

Results and discussion

Table 1 shows acetone control values with and without S9 from 7 experiments performed on dif-

TABLE 1

PROPHAGE INDUCTION IN THE PRESENCE OF ACETONE

Acetone (%)	S9	PFU/plate [a]							
		Expt. No.							$\bar{X} \pm$ S.D.
		1	2	3	4	5	6	7	
0.08	+	4	24	18	12	2	14	11	12.1 ± 7.6
0.16	+	6	12	18	12	3	11	13	10.7 ± 4.9
0.31	+	7	17	18	7	4	14	12	11.3 ± 5.4
0.63	+	12	21	28	20	5	20	13	17.0 ± 7.5
1.25	+	16	42	40	22	13	28	23	26.3 ± 11.2
2.50	+	20	46	44	18	12	40	21	28.7 ± 14.1
5.00	+	21	46	61	26	13	63	28	36.9 ± 19.9
10.00	+	30	54	59	32	21	50	38	40.6 ± 14.0
0.08	−	13	9	3	4	6	14	13	8.9 ± 4.6
0.16	−	12	9	3	4	7	14	7	8.0 ± 4.0
0.31	−	26	7	8	16	5	21	6	12.7 ± 8.3
0.63	−	29	12	4	17	7	22	6	13.9 ± 9.3
1.25	−	43	6	6	13	13	41	7	18.4 ± 16.4
2.50	−	29	13	10	14	20	41	18	20.7 ± 10.9
5.00	−	29	9	13	16	18	42	16	20.4 ± 11.3
10.00	−	22	13	17	32	20	40	16	22.9 ± 9.1

[a] Each value is the average of 2 plates.

ferent days. As can be seen, acetone induced a low, but real, dose-related increase in PFU/plate. Thus, a mean averaged over all of the doses of acetone may not be an appropriate solvent control value. Because of this dose-related response, a more appropriate solvent control is the value from an acetone well relative to its corresponding test-compound well (both wells contain the same percentage of acetone). Accordingly, we have subtracted each acetone control value from its corresponding test-compound value and expressed this difference as the induced PFU/plate (Table 2). In addition, we have calculated the fold increase at each dose to provide a further indication of the magnitude of the induction.

The highest mean PFU/plate produced by acetone (in the absence of test compound) occurred at the highest concentration used, 10% (Table 1). Therefore, our criterion for a positive response was a dose-related increase of induced PFU/plate that reached or exceeded the upper limit of the 99% confidence interval for acetone at this concentration. Based on the average PFU/plate produced by 10% acetone for all experiments reported here, the upper limit of the 99% confidence interval was 60 PFU/plate in the presence of S9 and 37 PFU/plate in the absence of S9.

The response for each compound based on this criterion is shown in Table 2. For most of the positive compounds, the dose that produced either 60 (+S9) or 37 (−S9) induced PFU/plate generally produced a 3- or 4-fold increase of PFU/plate for the treated compared to the corresponding acetone control (Table 2). Thus, our criterion for a positive response established a fold increase similar to that recommended by Rossman et al. (1985) for a positive response (3-fold).

As discussed by Rossman et al. (1984), clear wells (or wells that are only slightly turbid) are due to cytotoxicity and/or inhibition of cell growth by the test compound. The cytotoxicities of the pesticides are noted in Table 2 by a 'T' for clear wells and a 't' for slightly turbid wells. Consistent with the findings of Rossman et al. (1984, 1985), no (or few) phage were isolated from clear wells (Table 2). For captan and dichlorvos, which were cytotoxic and induced prophage, maximum induction occurred near cytotoxic/inhibitory concentrations (Table 2). A genotoxic mechanism (induction), as opposed to a cytotoxic

TABLE 2

INDUCTION OF PROPHAGE λ BY PESTICIDES AND POSITIVE CONTROLS

Compound	Dose (mM)	+S9				−S9			
		PFU/plate		Induced PFU/plate	Fold increase	PFU/plate		Induced PFU/plate	Fold increase
		Compound	Acetone			Compound	Acetone		
Captan	0.0003	9	4	5	2.3	31	14	17	2.2
	0.0005	6	6	0	1.0	32	14	18	2.3
	0.0010	15	7	8	2.1	57	21	36	2.7
	0.0021	23	12	11	1.9	78	22	56	3.5
	0.0042	64	16	48	4.0	139	41	98	3.4
	0.0083	182	20	162	9.1	0 (T)	41	0	–
	0.0167	595	21	574	28.3	0 (T)	42	0	–
	0.0333	3 (T)	30	0	–	0 (T)	40	0	–
Response			+				+		
Chlordane	0.76	9	11	0	–	21	13	8	1.6
	1.52	9	13	0	–	20	7	13	2.9
	3.05	9	12	0	–	43	6	37	7.2
	6.10	11	13	0	–	37	6	31	6.2
	12.20	19	23	0	–	47	7	40	6.7
	24.39	42	21	21	2.0	33	18	15	1.8
	48.78	67	28	39	2.4	170	16	154	10.6
	97.56	235 (P)	38	197	6.2	482 (P)	16	466	30.1
Response			+				+		
Dichlorvos	0.04	9	11	0	–	11	13	0	–
	0.07	18	13	5	1.4	10	7	3	1.4
	0.14	19	12	7	1.6	13	6	7	2.2
	0.28	31	13	18	2.4	18	6	12	3.0
	0.57	57	23	34	2.5	33	7	26	4.7
	1.14	91	21	70	4.3	62	18	44	3.4
	2.27	71	28	43	2.5	168	16	152	10.5
	4.55	32 (t)	38	0	–	1 (T)	16	0	–
Response			+				+		
p,p'-DDT	0.46	15	18	0	–	7	13	0	–
	0.89	13	18	0	–	9	12	0	–
	1.79	28	18	10	1.6	15	26	0	–
	3.57	32	28	4	1.1	23	29	0	–
	7.14	41	40	1	1.0	28	43	0	–
	14.29	59	44	15	1.3	34	29	5	1.2
	25.57	49 (P)	61	0	–	28 (P)	29	0	–
	57.14	54 (P)	59	0	–	29 (P)	22	7	1.3
Response			–				–		
Lindane	1.07	29	24	5	1.2	70 (P)	5	65	14.0
	2.17	35	12	23	2.9	80 (P)	6	74	13.0
	4.31	69	17	52	4.1	56 (P)	5	51	11.2
	8.62	104	21	83	5.0	31 (P)	9	22	3.4
	17.24	126 (P)	42	84	3.0	58 (P)	12	46	4.8
	35.48	131 (P)	46	85	2.8	63 (P)	13	50	4.8
	68.97	118 (P)	46	72	2.6	64 (P)	9	55	7.1
	137.93	85 (P)	54	31	1.6	34 (P)	13	21	2.6
Response			+				+		

TABLE 2 (continued)

Compound	Dose (mM)	+S9				−S9			
		PFU/plate		Induced PFU/plate	Fold increase	PFU/plate		Induced PFU/plate	Fold increase
		Compound	Acetone			Compound	Acetone		
Malathion	0.47	3	12	0	–	6	4	2	1.5
	0.95	5	12	0	–	6	4	2	1.5
	1.89	8	7	1	1.1	13	16	0	–
	3.79	17	20	0	–	17	17	0	–
	7.58	24	22	2	1.1	6	13	0	–
	15.15	23	18	5	1.3	13	14	0	–
	30.30	23	26	0	–	11	16	0	–
	60.61	17 (T)	32	0	–	0 (T)	32	0	–
Response			–				–		
Mirex	0.04	15	14	1	1.1	20	14	6	1.4
	0.07	15	11	4	1.4	18	14	4	1.3
	0.14	21 (P)	14	7	1.5	25 (P)	21	4	1.2
	0.28	21 (P)	20	1	1.1	28 (P)	22	6	1.3
	0.57	28 (P)	28	0	1.0	29 (P)	41	0	–
	1.14	37 (P)	40	0	–	39 (P)	41	0	–
	2.27	46 (P)	63	0	–	40 (P)	42	0	–
	4.55	45 (P)	50	0	–	33 (P)	40	0	–
Response			–				–		
Monuron	0.04	8	24	0	–	6	6	0	1.0
	0.08	8	12	0	–	8	7	1	1.1
	0.16	9	17	0	–	3	5	0	–
	0.31	28	21	7	1.3	11	7	4	1.6
	0.63	37	42	0	–	8	13	0	–
	1.25	28	46	0	–	0 (T)	20	0	–
	2.50	0 (T)	46	0	–	0 (T)	18	0	–
	5.00	0 (T)	54	0	–	0 (T)	20	0	–
Response			–				–		
Nitrofen	0.28					9	6	3	1.5
	0.56					9	7	2	1.3
	1.12					10	5	5	2.0
	2.23					11	7	4	1.6
	4.46	33	11	22	3.0	11	13	0	–
	8.93	52	13	39	4.0	14	20	0	–
	17.86	61	23	38	2.7	6 (t)	18	0	–
	21.43	122	21	101	5.8				
	28.57	108 (P)	28	80	3.9				
	35.71	27 (P)	38	0	–	0 (T,P)	20	0	–
Response			+				–		
Toxaphene	0.10	15	12	3	1.3	28	9	19	3.1
	0.19	19	12	7	1.6	67	9	58	7.4
	0.38	21	7	14	3.0	127	7	120	18.1
	0.76	64	20	44	3.2	219	12	207	18.3
	1.52	83	22	61	3.8	359	6	353	59.8
	3.05	173	18	155	9.6	794	13	781	61.1
	6.10	173	26	147	6.7	898	9	889	99.8
	12.20	604 (P)	32	572	18.9	2184 (P)	13	2171	168.0
Response			+				+		

TABLE 2 (continued)

Compound	Dose (mM)	+S9				−S9			
		PFU/plate		Induced PFU/plate	Fold increase	PFU/plate		Induced PFU/plate	Fold increase
		Compound	Acetone			Compound	Acetone		
2-AA	0.0002	43	23	20	1.9				
	0.0004	81	16	65	5.1				
	0.0008	167	13	154	12.8				
	0.0016	453	7	446	64.7				
	0.0032	1753	13	1740	134.8				
Response			+						
2-NF	0.011					42	14	28	3.0
	0.022					50	14	36	3.6
	0.045					57	21	36	2.7
	0.089					59	22	37	2.7
	0.179					104 (P)	41	63	2.5
	0.357					170 (P)	41	129	4.1
	0.714					209 (P)	42	167	5.0
	1.429					411 (P)	40	371	10.3
Response							+		

T, toxic (clear well); t, slightly toxic (slightly turbid well); P, precipitate.

mechanism (release of preformed phage), appears to account for the observed dose response produced by these two cytotoxic agents because the response occurs in the absence of cytotoxicity (cell survival data not shown).

A general definition of potency is the concentration of an agent required to produce a particular response. Using the linear portion of the dose–response curves (Fig. 1), we calculated the concentration (mM) required by each compound to produce an induced PFU/plate of 60 (+S9) or 37 (−S9), representing the upper limit of the 99% confidence interval for acetone at a concentration of 10%. Each of these values may be viewed as the minimum concentration required to produce a positive response. Based on these values, the compounds were then ranked from most to least potent (Table 3).

As illustrated in Fig. 1 and Table 3, the concentrations of the pesticides required to produce positive responses ranged over 6 orders of magnitude. The induction potencies of the pesticides in the presence of S9 ranked as follows: captan > dichlorvos > toxaphene > lindane > nitrofen > chlordane. Rankings were similar in the absence of S9, with captan remaining the most potent. We were unable to calculate a potency value for lindane in the absence of S9 because it did not

TABLE 3

RELATIVE POTENCIES OF PESTICIDES

Compound	Concentration (mM) required to produce an induced PFU/plate at the upper limit of the 99% confidence interval based on the acetone (10%) controls [a] (rank)	
	+S9	−S9
Captan	0.004 (1)	0.001 (1)
Dichlorvos	0.98 (2)	0.93 (3)
Toxaphene	1.26 (3)	0.13 (2)
Lindane	5.85 (4)	+ [b]
Nitrofen	12.88 (5)	−
Chlordane	46.74 (6)	16.72 (4)

[a] This is the concentration (mM) required to produce 60 induced PFU/plate (+S9) or 37 induced PFU/plate (−S9).

[b] Potency value incalculable due to nonlinear dose response (see text).

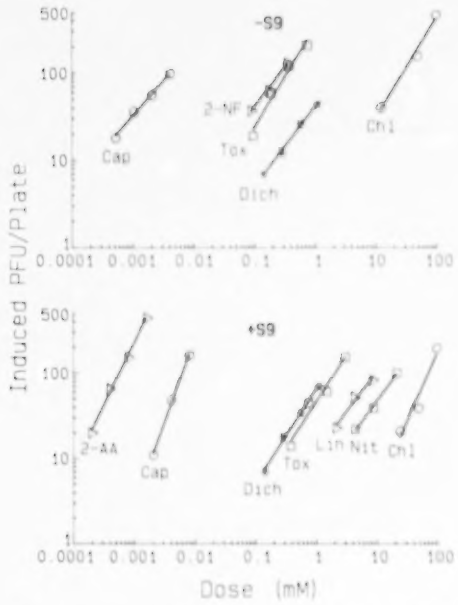

Fig. 1. Dose–response curves of prophage induction by pesticides and positive controls in the presence and absence of S9. Only the linear portions of the dose–response curves are shown, and the correlation coefficients (r^2 values) range from 0.94 to 0.99. Abbreviations: captan (Cap), toxaphene (Tox), dichlorvos (Dich), chlordane (Chl), lindane (Lin), nitrofen (Nit), 2-aminoanthracene (2-AA), and 2-nitrofluorene (2-NF).

produce a linear dose response, even though it was tested in 6 experiments over a wide range of doses (from 0.00003 mM to 137.9 mM). The absence of a dose-related response may have been related to the tendency of lindane to precipitate out of solution once added to the minimal medium (noted by a 'P' for precipitate in Table 2). Of all of the positive pesticides, only nitrofen required S9 to induce phage (Table 2).

Table 4 compares the pesticides with regard to their ability to induce prophage, their mutagenicity in Salmonella, and their carcinogenicity in rodents. All of the pesticides that were mutagenic in Salmonella also induced prophage. In addition, 2 carcinogens that are not mutagenic in Salmonella (chlordane and lindane) were detected by the phage-induction assay. 3 carcinogenic pesticides (p,p'-DDT, mirex, and monuron) were not detected by either assay, and one potential noncarcinogen (malathion) was negative in both microbial assays.

Although the data base is limited, these results begin to suggest that prophage induction may be a slightly more sensitive endpoint than mutagenesis in Salmonella for the detection of carcinogenic chlorinated organics. Based on these results and the need for an assay to complement the Salmonella assay for the detection of carcinogenic chlorinated organics, we are currently extending this study to include a broader selection of chlorinated compounds.

In addition to detecting carcinogenic chlorinated organics, the Microscreen phage-induction assay appears to detect genotoxic activity in more samples of industrial hazardous wastes than does

TABLE 4

COMPARISON OF PROPHAGE INDUCIBILITY, MUTAGENICITY, AND CARCINOGENICITY OF PESTICIDES

Pesticide	Response			References for Salmonella data
	Prophage induction	Salmonella	Carcinogenicity [a]	
Captan	+	+	LP	Kier et al., 1986
Chlordane	+	–	LP	Mortelmans et al., 1986
Dichlorvos	+	+	I	Kier et al., 1986
p,p'-DDT	–	–	SP	Kier et al., 1986
Lindane	+	–	LP	Haworth et al., 1983
Malathion	–	–	I(N)	Haworth et al., 1983
Mirex	–	–	SP	Mortelmans et al., 1986
Monuron	–	–	LP	Haworth et al., 1983
Nitrofen	+	+	SP	Dunkel et al., 1985
Toxaphene	+	+	SP	Mortelmans et al., 1986

[a] From Nesnow et al. (1987); SP, sufficient positive; LP, limited positive; I(N), inadequate (negative); I, inadequate.

the Salmonella assay (Houk and DeMarini, 1987). Thus, prophage induction may be useful for screening hazardous wastes, many of which contain chlorinated organics and carcinogenic solvents and metals that are refractory in the Salmonella assay. We plan to explore further the feasibility of the Microscreen phage-induction assay as a possible complement to the Salmonella assay for the detection of chlorinated carcinogens, carcinogenic solvents, and genotoxic complex mixtures.

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